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The long-term goal is to develop a new breast cancer treatment based on inhibition of the growth factor activity of procathepsin D. During the current year we tested all possible breast cancer cell lines for secretion of procathepsin D. Based on our data, we prepared numerous clones of MDA-MB-231 cells transfected with all expected vectors. Both the secretion of procathepsin D in vitro and growth activity in vivo of these clones has been established.

A library of 10 synthetic peptides with a single substitution of one amino acid have been prepared and tested. Based on this information we know the exact binding moiety on the molecule of activation peptide. We also prepared monoclonal antibodies against these peptides and tested them as potential inhibitors of breast cancer cell growth.

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INTRODUCTION

The long-term objective of this project is to develop a new treatment for breast cancer based on blockade of the autocrine growth factor activity of procathepsin D. Breast cancer cells secrete procathepsin D, the enzymatically inactive form from which the aspartic proteinase cathepsin D is generated by removal of an activation peptide (APpCD). Procathepsin D has been identified as an independent prognostic factor in several forms of cancer, particularly breast cancer. In preliminary experiments done by our group, procathepsin D was found to act as a specific autocrine growth factor for breast cancer-derived cells, but not for any other cell type tested. These effects were mediated through a specific receptor expressed on breast cancer cell lines that is distinct from the usually proposed cathepsin D-specific mannose-6-phosphate receptor. The region of procathepsin D responsible for its mitogenic activity was localized to APpCD and amino acids 27-44 of the APpCD sequence. No growth factor activity could be shown with the mature enzyme cathepsin D. The proposed specific aims are based on the central hypothesis that procathepsin D is involved in breast cancer via a specific receptor that mediates autocrine activation for increased metastatic growth. This project proposed the following aims: 1) it is hypothesized that the overproduction of procathepsin D results in an increase in the metastatic potential of breast tumor cells. A low metastatic human breast cancer cell line will be transfected with human procathepsin D cDNA such that the cells will secrete constitutively varying amounts of procathepsin D. The metastatic potential of each transfected cell line will be evaluated both *in vitro* and *in vivo* in relationship to the amount of procathepsin D secretion. 2) Attempts will be made to determine the exact site in procathepsin D responsible for breast cancer cell growth factor activity. Synthetic peptides representing fragments of APpCD will be prepared. Amino acid substitutions in the most active peptide fragment will be used to map the essential amino acid contact sites for the receptor. 3) It is hypothesized that inhibition of the APpCD interaction with its receptor will result in inhibition of cancer cell growth. Peptide analogs will be prepared with D-amino acids to block the growth and malignancy of cancer cells.

BODY

TASK 1

MDA-MD-231 cell line were transfected with various human procathepsin D cDNA and tested for secretion of procathepsin D *in vivo*. In addition, the growth pattern *in vivo* have been established. Preliminary results shown in Figures 1 and 2 (see Appendices) showed that the cells differ in their ability to growth *in vivo* based on level of procathepsin D secretion. Synthetic peptides with a single amino acid substations have been synthesized, subsequently, monoclonal antibodies against these peptides were prepared. Based on these preliminary data, we believe that the ***Task 1*** will be successfully achieved in next future.

TASK 3

Various cytokines are involved in both cancer development and in defense against cancer growth, and the exact role of individual cytokines remain unclear. In this part, we focused on the role of IL-4, IL-10 and IL-13 on procathepsin D-stimulated proliferation of breast cancer cells. Our results clearly showed that only ER^+ breast cancer cells responded to the presence of all three tested cytokines by proliferation, ER^- cells were resistant to the addition of cytokines (Figure 3). As addition of anti-procathepsin D antibodies blocked the growth potentiation of cancer cells, we can conclude that addition of these cytokines resulted in stimulation of synthesis and/or release of procathepsin D. This conclusion was further supported by findings of procathepsin D in culture supernatants of cells incubated with cytokines. These data are important for further attempts to inhibit procathepsin D production and secretion.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that the binding site of the procathepsin D activation peptide is located in the 27 – 44 AA region
- Establishment of all cell lines transfected with vectors containg human procathepsin D cDNA
- Establishment of basic values for invasiveness and *in vivo* correlation between procathepsin D production and cell growth *in vivo*
- Demonstration that invasiveness of breast cancer cells can be blocked by inhibition of procathepsin D secretion
- Demonstration of the exact binding site of activation peptide

REPORTABLE OUTCOMES

ABSTRACTS

I. **Vetvicka,V., Vetvickova, J., Fusek, M., Voburka, Z.:** Blocking of growth factor activity of procathepsin D inhibits human cancer, **Faseb J.,** Abstracts, p. A1000, 2000.

II. **Vetvicka, V., Vetvickova, J., Fusek, M., Voburka, Z.:** Inhibition of breast cancer by the procathepsin D activation peptide, **EACR XVI -2000 Meeting,** May 2000, Halkidiki, Greece, Abstract page 88.

III. **Vetvicka, V., Vetvickova, J., Voburka, Z., Fusek, M.:** Procathepsin activation peptide and its fragments in regulation of cancer growth, oral presentation, **13th International Symposium on Regulatory Peptides,** Cairns, Australia, October 22-26, 2000.

IV. **Vetvicka, V., Vetvickova, J., Fusek, M., Voburka, Z.:** Procathepsin D activation peptide and human cancer, AACR Meeting Molecular Biology and New Therapies in the 21st Century, Maui, February 12-16, 2001.

V. **Vetvicka, V., Vetvickova, J., Voburka, Z., Fusek, M.:** Procathepsin D stimulates the growth of human cancer – effect of cytokines. 92th Annual Meeting of American Association of Cancer Research, New Orleans 2001, p. 878

PAPERS

1. **Vetvicka,V., Vetvickova, J., Fusek,M.**: Role of procathepsin D activation peptide in prostate cancer development. **Prostate,** 44: 1-7, 2000

CONCLUSIONS

Based on results mentioned above, we have all reasons to believe that this project will be finished in successful and timely manner. Now when we established the exact binding site of activation peptide, we will focus our attention on Task 2 and isolation of the specific receptor.

As far as experiments in Task 3 are concerned, we already established that synthetic fragment corresponding to the 27-44 AA portion of activation peptide is responsible for binding of procathepsin D to the cancer cells. Subsequent experiments helped to locate the actual binding site to a region of 36-44 AA portion of the activation peptide. Further on, experiments using a library of synthetic peptides with a single amino acid substitutions helped establish the exact binding site on procathepsin D activation peptide.

In addition, we focused our attention on possible regulation of procathepsin D synthesis by cytokines. Various cytokines are involved in both cancer development and in defense against cancer growth, and the exact role of individual cytokines remain unclear. In this study, we focused on the role of IL-4, IL-10 and IL-13 on procathepsin D-stimulated proliferation of breast cancer cells. Our results clearly showed that only ER^+ breast cancer cells responded to the presence of all three tested cytokines by proliferation, ER^- cells were resistant to the addition of cytokines. As addition of anti-procathepsin D antibodies blocked the growth potentiation of cancer cells, we can conclude that addition of these cytokines resulted in stimulation of synthesis and/or release of procathepsin D. This conclusion was further supported by findings of procathepsin D in culture supernatants of cells incubated with cytokines.

So far, all obtained data support the original hypothesis that procathepsin D significantly stimulates the growth and spreading of breast cancer cells. If this hypothesis is further confirmed by this research project, this project has very significant potential to be developed into preclinical trials leading toward a new, very specific treatment of human breast cancer. In addition, recent observations by our and other groups suggest that the role of procathepsin D in human cancer development is probably even more general than we originally believed. In such a case, this project might be even more important.

Legend to the Figures

Figure 1

Comparison of the procathepsin D secretion in tissue culture supernatants of four cell clones (MDA-MB-231) transfected with vector containing cDNA coding human activation peptide. Using an ELISA assay employing anti-activation peptide antibodies, we compared the levels of procathepsin D secreted into tissue culture supernatants of transfected and parental cells. These data were confirmed by Western blotting.

Figure 2

The same cell clones were used for study of cell growth in vivo. Athymic nude mice were injected with 5×10^{6} cells ip. Ten days after injection, the mice were sacrificed, tumor excised and evaluated. The color codes correspond to the clones showed in Figure 1.

Figure 3

Growth of human breast cancer cells in serum-free medium containing different doses of human recombinant IL-10. After six days in culture, the proliferation was evaluated using an MTT assay. The results represent the mean of three independent experiments. The cytokine-stimulated growth of breast cancer cells correlates with the level of procathepsin D found in tissue culture supernatant (manuscript submitted).

Figure 1

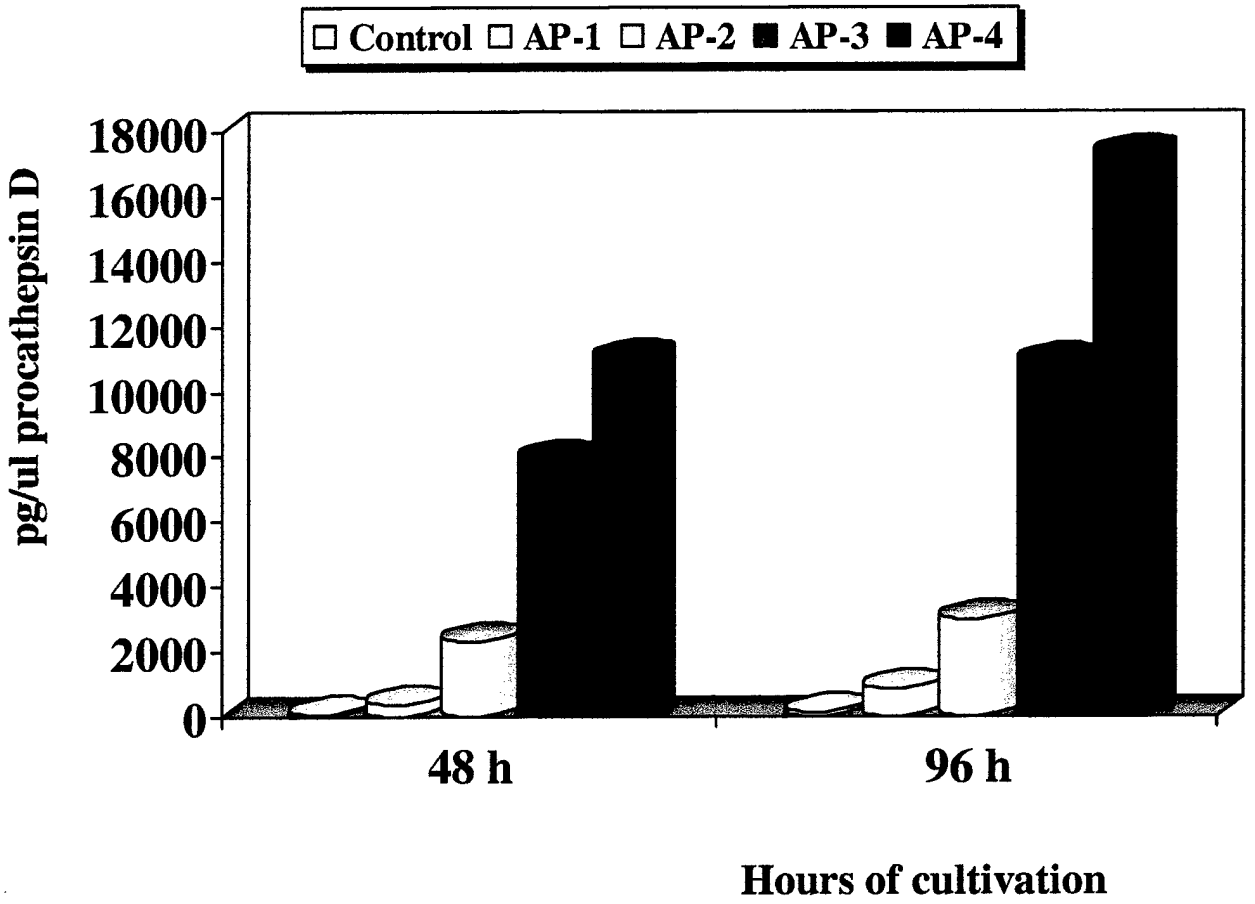

Control
AP-1
AP-2
AP-3
AP-4
pg/ul procathepsin D
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
48 h
96 h
Hours of cultivation

Figure 2

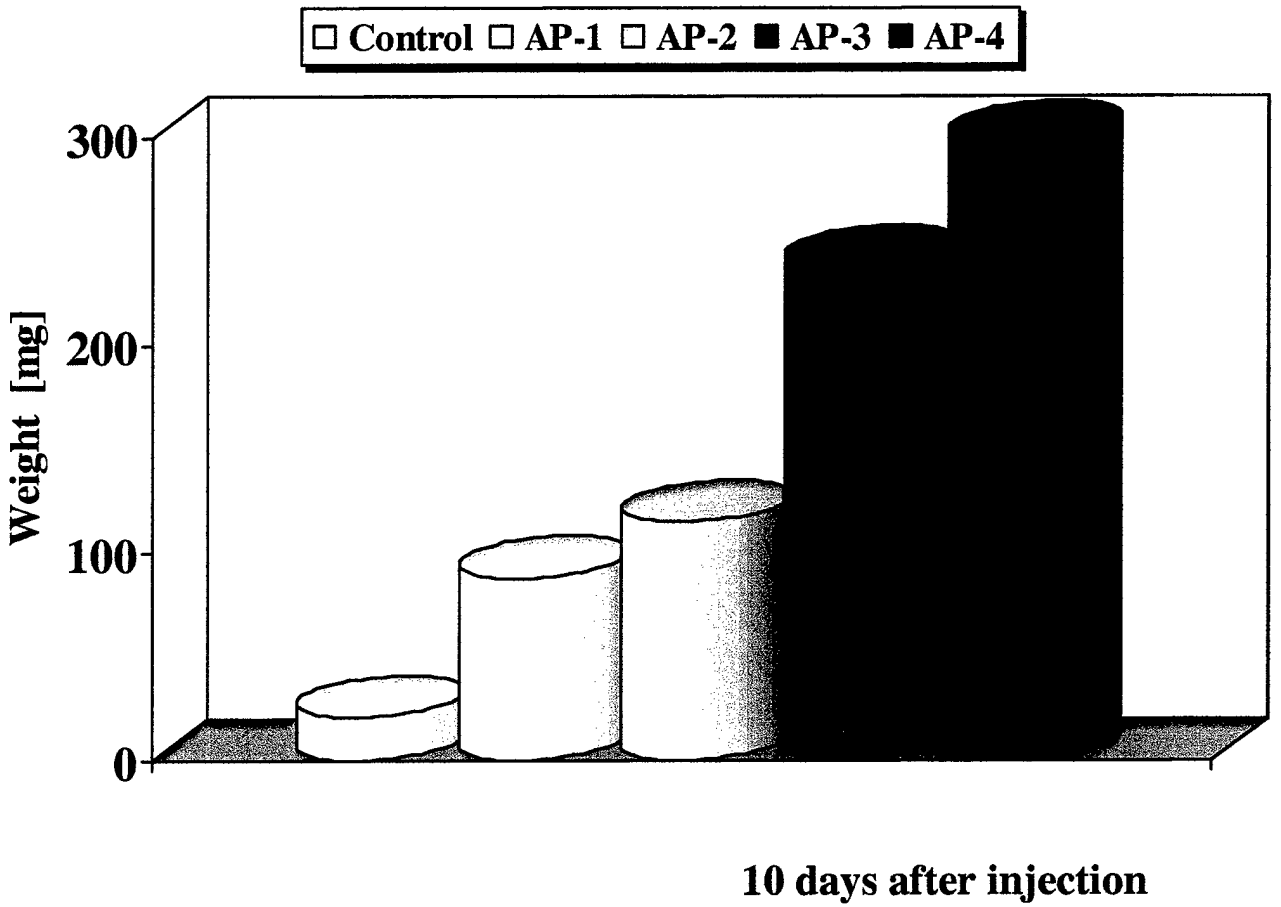
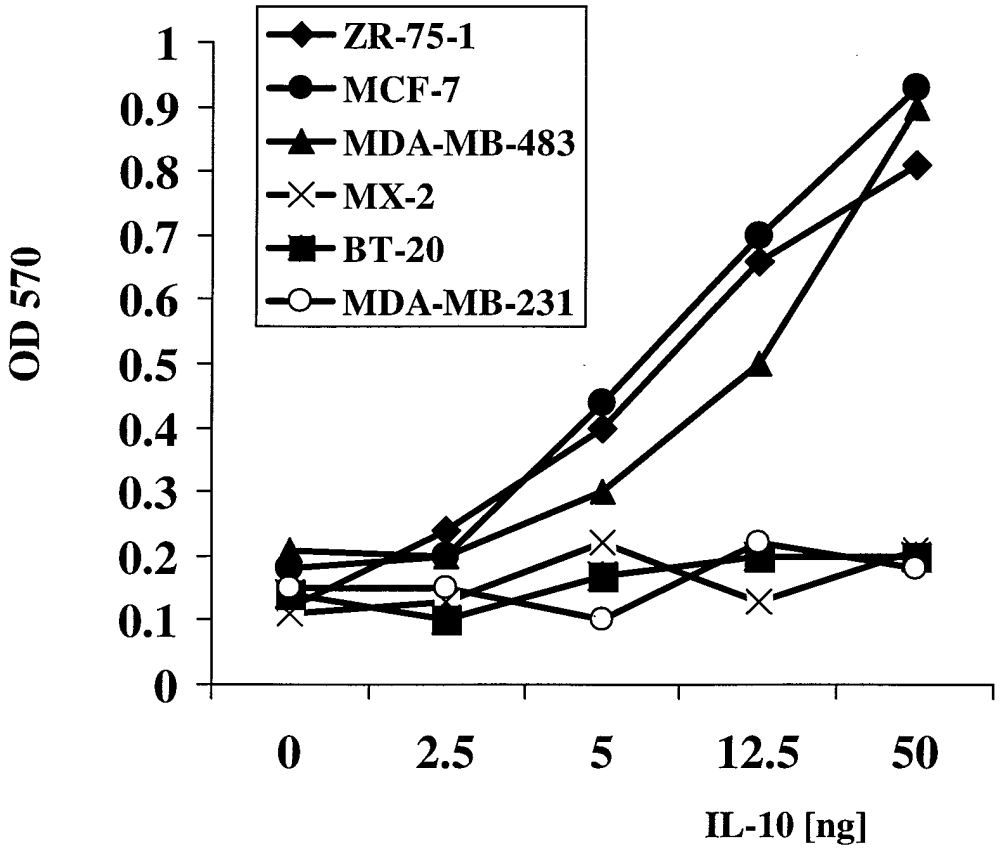

Control
AP-1
AP-2
AP-3
AP-4
300
200
100
0
Weight [mg]
10 days after injection

Figure 3


ZR-75-1
MCF-7
MDA-MB-483
MX-2
BT-20
MDA-MB-231
OD 570
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2.5
5
12.5
50
IL-10 [ng]